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Variation among goats in the ability of their polymorphonuclear neutrophil leukocytes and mammary secretions to support phagocytosis: inhibitory effects of milk fat globules

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Abstract

The objectives of this study were to determine if fat globules and casein in goat milk were inhibitory to phagocytosis by polymorphonuclear neutrophils (PMN) isolated from blood, and to determine if variation existed among goats in the ability of PMN to phagocytose and in the ability of milk whey to support phagocytosis. Milk and blood were obtained from six goats. Serum, skimmed milk and milk whey was prepared by centrifugation. In the presence of either serum, whole milk, skimmed milk or milk whey, PMN were incubated with fluorescein isothiocyanate-labeled *Staphylococcus aureus* in a 1:20 ratio. Phagocytosis of *S. aureus* was determined by flow cytometry. Both pooled serum and pooled whey had a concentration-dependent effect on PMN phagocytosis ($P < 0.01$). In the presence of pooled serum, whole milk but not skimmed milk reduced ($P < 0.01$) phagocytosis when compared to serum and whey. Differences existed ($P < 0.01$) among goats in the ability of PMN to phagocytose and in the ability of milk whey to support phagocytosis. The inhibition of PMN phagocytosis by fat globules could contribute to susceptibility to intramammary infection by mastitis pathogens. Variation among goats in PMN phagocytosis and in mammary secretions to support phagocytosis may contribute to differences among goats in resistance to mastitis.

Keywords: Goats; Phagocytosis; Neutrophil; Mastitis

1. Introduction

Phagocytosis of bacteria by polymorphonuclear neutrophil leukocytes (PMN) is a major defense against intramammary infections by mastitis pathogens. In dairy cows, PMN isolated from milk are less phagocytic than PMN isolated from blood (Kent and Newbould, 1969; Russell and Reiter, 1975). Studies of in vitro phagocytosis indicate that the milk fat globules and casein in cow milk exert inhibitory effects on phagocytosis (Paape et al., 1975; Paape and Guidry, 1977). Transmission electron microscopy revealed the presence of milk fat globules and casein within vacuoles of bovine PMN (Paape and Wergin, 1977). Also, ingestion by PMN of particles, such as milk fat globules and casein, cause a loss of cytoplasmic granules, inhibit depression of pH within phagosomes, which are associated with a reduction in bactericidal activities, and result in leukocyte rounding, which eliminate pseudopods needed for phagocytic capabilities (Paape

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and Guidry, 1977; Paape et al., 1981; Reinitz et al., 1982). It was also observed that significant variation existed among cows in the ability of bovine PMN to phagocytose and in milk to support phagocytosis of *Staphylococcus aureus* (Paape et al., 1978; Paape and Pearson, 1979). Similar studies on possible inhibitory effects of milk components on phagocytosis by goat PMN, and variation among goats in the ability of their PMN to phagocytose and in milk whey to support phagocytosis have not been performed. Such information would be important in better understanding susceptibility of the mammary gland of goats to mastitis and understanding differences among goats in susceptibility to infection.

The objectives of this study were to determine: (1) if milk fat globules and casein were inhibitory to the phagocytosis of *S. aureus* by PMN isolated from goat blood, and (2) if variation existed among goats in the ability of their PMN to phagocytose and in the ability of their milk whey to support phagocytosis of *S. aureus* by PMN.

2. Materials and methods

2.1. Goats

Six clinically normal Nubian goats were selected for study. Goats were in their fifth month of lactation and free from intramammary infection by major and minor mastitis pathogens. Use of animals for this investigation was approved by the Beltsville Agricultural Research Center Animal Care and Use Committees.

2.2. Preparation of serum, whole milk, skimmed milk and milk whey

Jugular vein blood was collected from six goats and allowed to clot at room temperature overnight. The serum was collected by centrifugation at 46,000 × *g* for 20 min at 4 °C. A 10 ml portion of serum from each of the six goats was pooled, aliquoted, and frozen at −20 °C. The remaining serum was aliquoted and frozen at −20 °C.

Whole milk was collected from mixed bucket milk after machine milking. A portion was centrifuged at 6500 × *g* and the cream layer removed. The sample was recentrifuged and the residual cream was removed by aspiration. The sample was decanted carefully to avoid disturbing the sediment, aliquoted, and frozen at −20 °C. A portion of the skimmed milk was centrifuged at 46,000 × *g* to remove the casein. The whey was aliquoted and frozen at −20 °C.

2.3. Preparation of bacteria

S. aureus (Newbould strain 305) were heat killed and adjusted to 1×10^9 cells/ml. Bacteria were stained with fluorescein isothiocyanate (FITC) according to the method of Gelfand et al. (1976).

2.4. Isolation of blood PMN

For the isolation of blood PMN, 30 ml samples of jugular vein blood were collected in 40 ml (partial vacuum) tubes that contained 3 ml of acid–citrate–dextrose solution formula A (33.44 g of dextrose, 19.98 g of citric acid, and 33 g of sodium citrate in 1000 ml of water). The PMN were isolated by differential centrifugation and hypotonic lysis of the red blood cells according to the procedure of Carlson and Kaneko (1973). The only exceptions were that the entire isolation was performed at 4 °C, and the glassware and stoppers were siliconized. The cell pellet was resuspended in 10 ml of sterile 0.01 M phosphate-buffered 0.85% saline, pH 7.4 (PBS). Cells were counted with an electronic cell counter (Coulter Electronics, Hialeah, FL). Differential leukocyte counts were determined microscopically on 100 cells on each of two Wright-stained smears. Viability was determined using 0.1% trypan blue. Purity and viability of the isolated PMN was >90 and >95%, respectively. The final leukocyte suspension was adjusted to 10×10^6 viable PMN/ml.

2.5. Phagocytosis assay

Phagocytosis by PMN was measured using a Coulter EPICS Profile flow cytometer (Coulter Cytometry, Hialeah, FL) equipped with a 488 nm argon ion laser using the procedure of Saad and Hageltorn (1985). All of the phagocytosis assays were run in duplicate and consisted of 100 μl PMN (1×10^6 cells), 20 μl FITC-labeled *S. aureus* (20×10^6 cells), and either PBS, blood serum, whole milk, milk whey or skimmed milk in a final volume of 2 ml in 4 ml plastic vials. The

mixture was allowed to incubate with gentle rocking at 39 °C for 30 min and placed on ice. The samples were analyzed by flow cytometry before quenching the extracellular fluorescence of bacteria attached to the PMN cell surface and bacteria suspended in the media with 1% methylene blue, and after quenching to determine the percentage of PMN with only phagocytosed *S. aureus*. The final methylene blue concentration in each vial was 0.2%.

2.6. Effect of concentration of blood serum and milk whey on phagocytosis

To determine the effect of blood serum and milk whey concentration on phagocytosis, the assay consisted of mixing PMN and bacteria in vials with either 0, 10, 25, 50, 100 or 200 μl of either pooled blood serum or milk whey, and brought up to 2 ml with PBS. This provided serum and whey concentrations in the incubation mixture of 0, 0.50, 1.25, 2.50, 5 and 10%. A total of two runs were conducted. Each run was performed on a different day using PMN isolated from three different goats.

2.7. Effect of milk fat globules and casein on phagocytosis

To determine the effects of milk fat globules and casein on phagocytosis of *S. aureus* by PMN, the assay consisted of mixing PMN, bacteria and 200 μl of pooled blood serum with 1600 μl of either PBS, whole milk, skimmed milk or milk whey. A total of six runs were conducted. Each run was performed on a different day using PMN isolated from a different goat. On the day of a run, milk was collected from the goat serving as the PMN donor for the preparation of whole milk, skimmed milk and whey.

2.8. Variation among goats in milk whey to support phagocytosis and PMN to phagocytose

Variation among the six goats in the ability of their milk whey to support phagocytosis was determined using 200 μl of whey from each goat and PMN isolated from one goat. Six runs were performed on different days using PMN isolated from a different goat on each day.

Variation among the six goats in the ability of their PMN to phagocytosis *S. aureus* was determined using 200 μl of either pooled serum or pooled whey. A days run consisted in using PMN isolated from three different goats for a total of two runs.

2.9. Statistical analysis

Each experiment was analyzed as a randomized complete block split plot design, using mixed model procedures (SAS Institute Inc., Cary, NC).

3. Results

Increasing concentrations of blood serum resulted in both an increase ($P < 0.01$) in associated *S. aureus* (attached and phagocytosed) and phagocytosed *S. aureus* (Fig. 1). When compared to a concentration of 0% serum, attachment of bacteria to the PMN cell wall occurred at a serum concentration of 2.5% ($P < 0.05$). Phagocytosis occurred at a serum concentration of 5% ($P < 0.05$). Maximum attachment and phagocytosis occurred at a serum concentration of 10% serum. The correlation between associated and phagocytosed *S. aureus* in serum was 0.96.

Increasing concentrations of milk whey resulted in an increase ($P < 0.01$) in both associated and phagocytosed *S. aureus* (Fig. 2). When compared to 0% milk whey in the incubation mixture, a whey concentration of 1.25% was required for attachment of bacteria to PMN ($P < 0.05$) and 2.5% was required for phagocytosis ($P < 0.05$). Maximum attachment of bacteria to PMN occurred at a whey concentration of 5%. Maximum phagocytosis occurred at a whey concentration of 10%. The correlation between associated and phagocytosed *S. aureus* in whey was 0.98. When the quenched results in Figs. 1 and 2 were compared, serum and whey were different ($P < 0.01$), with whey supporting a higher degree of phagocytosis compared to serum. There was no difference ($P > 0.05$) in the unquenched results.

Both whole milk and skimmed milk reduced ($P < 0.01$) the percentage of PMN with associated *S. aureus* when compared to serum or whey (Table 1). There was no difference ($P < 0.05$) between serum and milk whey in the percentage of PMN with associated *S. aureus*. Whole milk but not skimmed milk

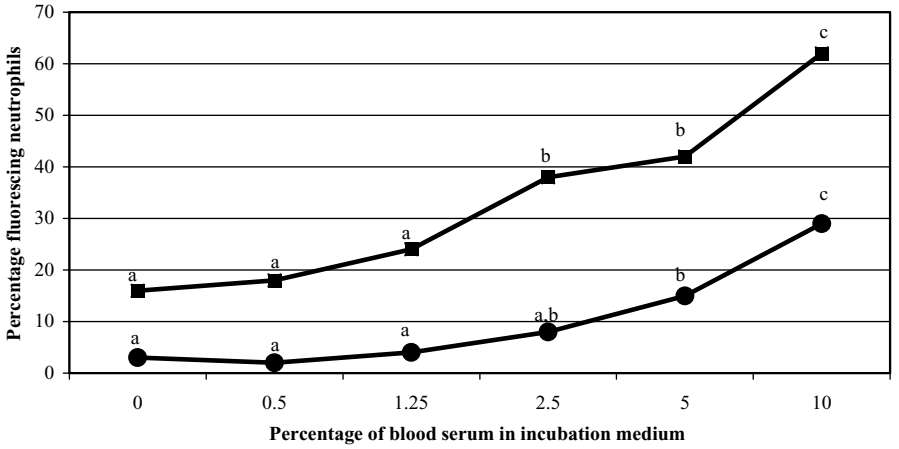

Fig. 1. Influence of the concentration of blood serum on the percentage of polymorphonuclear neutrophil leukocytes (PMN) with associated (attached and phagocytosed) and phagocytosed *S. aureus*. (■) Associated *S. aureus*, (●) phagocytosed *S. aureus*. Means not sharing a common letter (a–c) within a line are different ($P < 0.05$).

reduced ($P < 0.01$) the percentage of PMN with phagocytosed *S. aureus*. While the percentage phagocytosis of PMN in skimmed milk was less than that observed for whey (48% versus 60%) the difference was not significant ($P > 0.05$).

Variation existed ($P < 0.01$) among goats in the ability of their milk whey to support phagocytosis of *S. aureus* by isolated blood PMN and also in the percentage of PMN with associated *S. aureus* (Table 2). Neutrophils incubated with *S. aureus* in whey prepared from milk from goat #6 had more associated (80%, $P < 0.05$) and phagocytosed (57%, $P < 0.05$) *S. aureus* than did PMN incubated in whey prepared from milk of the other five goats.

Variation also existed ($P < 0.01$) among goats in the percentage of PMN with associated and phagocytosed *S. aureus* (Table 3). There were more PMN with associated and phagocytosed *S. aureus* in milk whey than in blood serum ($P < 0.01$). The percentage of PMN with associated *S. aureus* in serum and whey

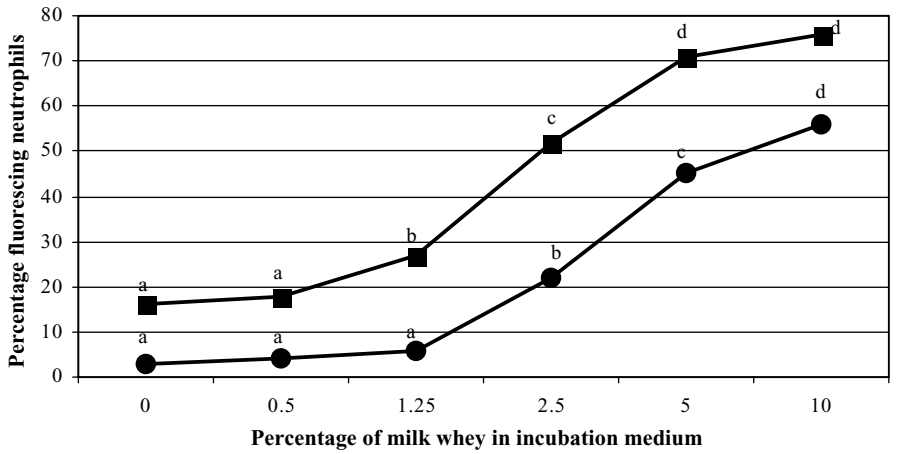

Fig. 2. Influence of the concentration of milk whey on the percentage of polymorphonuclear neutrophil leukocytes (PMN) with associated (attached and phagocytosed) and phagocytosed *S. aureus*. (■) Associated *S. aureus*, (●) phagocytosed *S. aureus*. Means not sharing a common letter (a–d) within a line are different ($P < 0.05$).

Table 1
Effect of studied material on the percentage of neutrophils (PMN) with associated (attached and phagocytosed) and phagocytosed *S. aureus*

Studied material[a]	PMN[b] (%)	
	Associated	Phagocytosed
Serum	77 a	58 a
Whole milk	44 b	41 bc
Skimmed milk	53 b	48 ac
Milk whey	73 a	60 a
SEM	±6	±6

Means within the column not sharing a common letter (a–c) are different ($P < 0.01$).

[a] Each value represents the mean from six runs using PMN isolated from blood of a different goat.

[b] Incubation mixture consisted of 20 μl *S. aureus* (20×10^6 cells), 100 μl pooled goat serum and 1680 μl of studied material.

Table 2
Effect of milk whey from different goats on the percentage of neutrophils (PMN) with associated (attached and phagocytosed) and phagocytosed *S. aureus*

Goat[a]	PMN[b] (%)	
	Associated	Phagocytosed
1	68 ab	39 ab
2	60 a	31 a
3	70 b	44 bc
4	64 ab	38 ac
5	64 ab	40 bc
6	80 c	57 d
SEM	±6	±6

Means within the column not sharing a common letter (a–d) are different ($P < 0.05$).

[a] Each value represents the mean from six runs using PMN isolated from blood of a different goat.

[b] Incubation mixture consisted of 20 μl *S. aureus* (20×10^6 cells), 100 μl PMN, 200 μl whey and 1680 μl PBS.

averaged 33 and 44%, and for phagocytosed *S. aureus* they averaged 10 and 23%.

4. Discussion

An unexpected finding from the results that examined the effects of increasing concentration of blood serum and milk whey on phagocytosis was that there were more PMN with associated and phagocytosed

Table 3
Variation in the percentage of neutrophils (PMN) isolated from blood of six different goats with associated (attached and phagocytosed) and phagocytosed *S. aureus* when incubated in either pooled serum or pooled whey

Goat[a]	PMN[b] (%)			
	Pooled serum		Pooled whey	
	Associated	Phagocytosed	Associated	Phagocytosed
1	31 a	9 a	40 a	21 ab
2	34 bc	10 ab	43 b	20 b
3	35 b	9 ab	46 cf	24 ac
4	31 a	10 ab	41 d	24 ac
5	33 ac	12 b	46 ce	26 cd
6	36 b	11 ab	45 f	21 ab
SEM	±0.9	±1.2	±0.3	±1.5

Means within the column not sharing a common letter (a–f) are different ($P < 0.05$).

[a] Each value represents the mean of two runs.

[b] Incubation mixture consisted of 20 μl *S. aureus* (20×10^6 cells), 100 μl PMN (1×10^6 cells), 200 μl of whey or serum and 1680 μl PBS.

S. aureus when incubated in milk whey compared to PMN incubated in blood serum. This was also observed in the experiment that examined the variation among goats in ability of PMN to phagocytose where either serum or milk whey was used as the incubation media. Bovine milk whey and blood serum have been reported to support similar levels of phagocytosis of *S. aureus* when using bovine PMN isolated from either blood or milk (Paape et al., 1975). Concentrations of IgM and IgG_2, opsonizing antibodies for ruminant PMN, have been reported (Butler, 1981) to be in higher concentrations in serum (3.69 and 9.04 mg/ml) than in milk (0.04 and 0.05 mg/ml). One would assume that with higher concentrations of opsonins a greater degree of phagocytosis would occur when serum was used in the incubation media for opsonization of bacteria. Immunoglobulin concentrations in pooled serum and whey used in the present study were not determined. It is possible that during the 30 min incubation at 37 °C that a portion of the immunoglobulins in serum may have formed aggregates and were unable to properly opsonize the bacteria. Heating at 60 °C for 30 min will cause complete aggregation of IgG_1 and IgG_2 (Leino and Paape, 1996).

We also observed that PMN with associated *S. aureus* occurred in lower concentrations of serum and

whey when compared to PMN with phagocytosed *S. aureus*. This suggests that adherence of *S. aureus* to the PMN surface preceded phagocytosis. The high correlation between PMN with associated bacteria and PMN with phagocytosed bacteria indicate that once bacteria become attached to the surface of PMN internalization will occur. This observation supports use of opsonized zymosan particles and PMN in chemiluminescence assays that are often used as an index of phagocytosis (Magnusson and Greko, 1991). In these assays, adherence of zymosan to the PMN surface triggers a chemiluminesence response (Leino and Paape, 1993).

Similar to what has been reported in dairy cows, cream in goat milk inhibited phagocytosis of *S. aureus* by PMN (Paape et al., 1975). However, the degree of inhibition in phagocytosis was greater in cow milk (1.8-fold) than it was in this study for goat milk (1.4-fold). While the fat content of goat milk is higher in goat milk (4.5%) compared to cow milk (3.7%), the fat globules in goat milk are considerably smaller and more fragile than fat globules in cow milk (Jenness, 1980). This may have contributed to them being less inhibitory to phagocytosis than fat globules in cow milk. In the present study, while casein in the skimmed milk caused a reduction in the percentage of PMN with associated bacteria it did not significantly reduce phagocytosis as was reported for bovine PMN (Paape et al., 1975; Paape and Guidry, 1977). The casein concentration in goat milk (2.5%) is slightly lower than cow milk (2.8%) and may have contributed to the lack of effect of casein on phagocytosis for goat milk (Jenness, 1980). Electron micrographs of bovine milk PMN revealed the presence of phagocytosed casein micelles, fat globules, and fat globule membrane material that was associated with peroxidase-positive material from azurophilic granules (Paape and Wergin, 1977; Dulin et al., 1988). The first line of defense against bacteria once they have passed the barrier of the streak canal are PMN. The inhibitory effect of fat globules on phagocytosis by PMN demonstrated in the present study, could increase the susceptibility of the goat mammary gland to intramammary infection.

Variation among goats in the ability of their milk whey to support phagocytosis and in their PMN to phagocytose was an important finding from the present study. Such variation could contribute to variation among goats in susceptibility to intramammary infection. Dairy cows whose milk supported a high level of phagocytosis were more resistant to mastitis following either a natural or experimental challenge of mastitis pathogens (Paape et al., 1978; Guidry et al., 1980).

5. Conclusions

Milk fat globules but not casein inhibited phagocytosis of *S. aureus* by goat PMN isolated from blood. Ingestion of milk fat globules by PMN may contribute to susceptibility of the gland to infection. Significant variation existed among goats in the ability of their PMN to phagocytose, and of their milk whey to support phagocytosis of *S. aureus* by PMN. This variation may be useful in selecting goats for mastitis resistance.

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